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FORM-PTO-1390 (Rev. 9-2001)		U S DEPARTMENT OF COMMERCE PATENT AND TRADEMARK OFFICE	ATTORNEY'S DOCKET NUMBER 017753-160
<b>TRANSMITTAL LETTER TO THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US) CONCERNING A FILING UNDER 35 U.S.C. 371</b>			U S APPLICATION NO (If known, see 37 C.F.R. 1.5) UNASSIGNED <b>10/089803</b>
INTERNATIONAL APPLICATION NO. PCT/FROO/02753	INTERNATIONAL FILING DATE 4 OCTOBER 2000	PRIORITY DATE CLAIMED 5 OCTOBER 1999	
TITLE OF INVENTION METHOD FOR PREPARING STEROIDS MODIFIED BY YEAST FERMENTATION			
APPLICANT(S) FOR DO/EO/US Gilles CAUET; Eric DEGRYSE; Pedro VICO and Richard LATHE			
Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:			
1. <input checked="" type="checkbox"/> This is a <b>FIRST</b> submission of items concerning a filing under 35 U.S.C. 371. 2. <input type="checkbox"/> This is a <b>SECOND</b> or <b>SUBSEQUENT</b> submission of items concerning a filing under 35 U.S.C. 371. 3. <input checked="" type="checkbox"/> This is an express request to begin national examination procedures (35 U.S.C. 371(f)). The submission must include items (5), (6), (9) and (21) indicated below. 4. <input checked="" type="checkbox"/> The US has been elected by the expiration of 19 months from the priority date (Article 31). 5. <input checked="" type="checkbox"/> A copy of the International Application as filed (35 U.S.C. 371(c)(2)) a. <input type="checkbox"/> is attached hereto (required only if not communicated by the International Bureau). b. <input checked="" type="checkbox"/> has been communicated by the International Bureau. c. <input type="checkbox"/> is not required, as the application was filed in the United States Receiving Office (RO/US). 6. <input checked="" type="checkbox"/> An English language translation of the International Application as filed (35 U.S.C. 371(c)(2)) a. <input checked="" type="checkbox"/> is attached hereto. b. <input type="checkbox"/> has been previously submitted under 35 U.S.C. 154(d)(4). 7. <input checked="" type="checkbox"/> Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371(c)(3)) a. <input type="checkbox"/> are attached hereto (required only if not communicated by the International Bureau). b. <input checked="" type="checkbox"/> have been communicated by the International Bureau. c. <input type="checkbox"/> have not been made; however, the time limit for making such amendments has NOT expired. d. <input type="checkbox"/> have not been made and will not be made. 8. <input checked="" type="checkbox"/> An English language translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371(c)(3)). 9. <input type="checkbox"/> An oath or declaration of the inventor(s) (35 U.S.C. 371(c)(4)). 10. <input type="checkbox"/> An English language translation of the annexes to the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371(c)(5)). 			
<b>Items 11 to 20 below concern document(s) or information included:</b> 11. <input type="checkbox"/> An Information Disclosure Statement under 37 CFR 1.97 and 1.98. 12. <input type="checkbox"/> An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included. 13. <input checked="" type="checkbox"/> A <b>FIRST</b> preliminary amendment. 14. <input type="checkbox"/> A <b>SECOND</b> or <b>SUBSEQUENT</b> preliminary amendment. 15. <input type="checkbox"/> A substitute specification. 16. <input type="checkbox"/> A change of power of attorney and/or address letter. 17. <input checked="" type="checkbox"/> A computer-readable form of the sequence listing in accordance with PCT Rule 13ter.2 and 35 U.S.C. 1.821 - 1.825. 18. <input type="checkbox"/> A second copy of the published international application under 35 U.S.C. 154(d)(4). 19. <input type="checkbox"/> A second copy of the English language translation of the international application under 35 U.S.C. 154(d)(4). 20. <input checked="" type="checkbox"/> Other items or information: 			
Form PCT/RO/101 (4 pgs.); Verification of Translation of Amended Sheets (3 sheets); Form PCT/IB/301; Form PCT/IB/304; Form PCT/IPEA/409 and English Translation (5 pgs.); Form PCT/IPEA/416; Form PCT/IB/308 and (2) Sheets of Sequence Listing.			



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1C10 Rec'd PCT/PTO 0 4 APR 2002

U.S. APPLICATION NO (If known, see 37 CFR 1.55) <b>UNASSIGNED</b>		INTERNATIONAL APPLICATION NO <b>PCT/FRO0/02753</b>		ATTORNEY'S DOCKET NUMBER <b>017753-160</b>	
21. <input checked="" type="checkbox"/> The following fees are submitted:				<b>CALCULATIONS</b>	PTO USE ONLY
<b>Basic National Fee (37 CFR 1.492(a)(1)-(5)):</b>  Neither international preliminary examination fee (37 CFR 1.482) nor international search fee (37 CFR 1.445(a)(2)) paid to USPTO and International Search Report not prepared by the EPO or JPO ..... \$1,040.00 (960)  International preliminary examination fee (37 CFR 1.482) not paid to USPTO but International Search Report prepared by the EPO or JPO ..... \$890.00 (970)  International preliminary examination fee (37 CFR 1.482) not paid to USPTO but international search fee (37 CFR 1.445(a)(2)) paid to USPTO ..... \$740.00 (958)  International preliminary examination fee (37 CFR 1.482) paid to USPTO but all claims did not satisfy provisions of PCT Article 33(1)-(4) ..... \$710.00 (956)  International preliminary examination fee (37 CFR 1.482) paid to USPTO and all claims satisfied provisions of PCT Article 33(1)-(4) ..... \$100.00 (962)  <div style="text-align: right;"><b>ENTER APPROPRIATE BASIC FEE AMOUNT =</b></div>				<div style="text-align: right;"><b>\$ 890.00</b></div>	
Surcharge of <b>\$130.00 (154)</b> for furnishing the oath or declaration later than months from the earliest claimed priority date (37 CFR 1.492(e)).      20 <input type="checkbox"/> 30 <input type="checkbox"/>					
Claims	Number Filed	Number Extra	Rate		
Total Claims	17 -20 =	0	X\$18.00 (966)	\$ 0.00	
Independent Claims	3 -3 =	0	X\$84.00 (964)	\$ 0.00	
Multiple dependent claim(s) (if applicable)			+ \$280.00 (968)	\$ 0.00	
<b>TOTAL OF ABOVE CALCULATIONS =</b>				<b>\$ 890.00</b>	
Reduction for 1/2 for filing by small entity, if applicable (see below).      +				\$	
<b>SUBTOTAL =</b>				<b>\$ 890.00</b>	
Processing fee of <b>\$130.00 (156)</b> for furnishing the English translation later than months from the earliest claimed priority date (37 CFR 1.492(f)).      20 <input type="checkbox"/> 30 <input type="checkbox"/>				\$	
<b>TOTAL NATIONAL FEE =</b>				<b>\$ 890.00</b>	
Fee for recording the enclosed assignment (37 CFR 1.21(h)). The assignment must be accompanied by an appropriate cover sheet (37 CFR 3.28, 3.31). <b>\$40.00 (581)</b> per property      +				\$	
<b>TOTAL FEES ENCLOSED =</b>				<b>\$ 890.00</b>	
				<b>Amount to be refunded:</b>	\$
				<b>charged:</b>	\$
<p>a. <input type="checkbox"/> Small entity status is hereby claimed.</p> <p>b. <input checked="" type="checkbox"/> A check in the amount of \$ <u>890.00</u> to cover the above fees is enclosed.</p> <p>c. <input type="checkbox"/> Please charge my Deposit Account No. <u>02-4800</u> in the amount of \$ _____ to cover the above fees. A duplicate copy of this sheet is enclosed.</p> <p>d. <input checked="" type="checkbox"/> The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any overpayment to Deposit Account No. <u>02-4800</u>. A duplicate copy of this sheet is enclosed.</p> <p><b>NOTE: Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137(a) or (b)) must be filed and granted to restore the application to pending status.</b></p> <p>SEND ALL CORRESPONDENCE TO:</p> <p>Norman H. Stepno, Esq. BURNS, DOANE, SWECKER &amp; MATHIS, L.L.P. P.O. Box 1404 Alexandria, Virginia 22313-1404 (703) 836-6620</p>					
SIGNATURE  <b>TERESA STANEK REA</b> NAME				APRIL 4, 2002 DATE	
30,427 REGISTRATION NUMBER					

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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Application No. :

U.S. National Serial No. :

Filed :

PCT International Application No. : PCT/FR00/02753

VERIFICATION OF A TRANSLATION

I, the below named translator, hereby declare that:

My name and post office address are as stated below;

That I am knowledgeable in the French language in which the below identified international application was filed, and that, to the best of my knowledge and belief, the English translation of the international application No. PCT/FR00/02753 is a true and complete translation of the above identified international application as filed.

I hereby declare that all the statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the patent application issued thereon.

Date: March 15, 2002

Full name of the translator :

  
Elaine Patricia PARRISH

For and on behalf of RWS Group plc

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10/089803

JC10 Rec'd PCT/PTO 04 APR 2002  
Patent

Attorney's Docket No. 017753-160

**IN THE UNITED STATES PATENT AND TRADEMARK OFFICE**

In re Patent Application of	)	
	)	
Gilles CAUET <i>et al.</i>	)	Group Art Unit: Unassigned
	)	
Application No.: Unassigned	)	Examiner: Unassigned
(Corresponds to PCT/FR00/02753)	)	
	)	
International Filing Date: 4 October 2000	)	
	)	
For: <b>METHOD FOR PREPARING</b>	)	
<b>STERIODS MODIFIED BY YEAST</b>	)	
<b>FERMENTATION</b>	)	
	)	

**PRELIMINARY AMENDMENT**

**BOX PCT**  
Assistant Commissioner for Patents  
Washington, D.C. 20231

Sir:

Prior to examination, please amend the above-captioned application as follows:

**IN THE CLAIMS:**

Please cancel claims 1-16 without prejudice or disclaimer and insert the following new claims in lieu thereof.

--17. A method for producing hydroxylated and/or acetylated steroids, comprising the steps according to which:

- yeasts are cultured on a medium comprising at least one precursor of such hydroxylated and/or acetylated steroids, and then

- the hydroxylated and/or acetylated steroids are isolated from the medium after bioconversion,

wherein said yeasts are yeasts transformed so as to express the product of the *Cyp7b* gene.

**18.** The method of claim 17, for producing a hydroxylated steroid, wherein said yeasts have low or zero APAT activity.

**19.** The method of claim 17, for producing a hydroxylated steroid, wherein said yeasts are cultured under conditions which are oxidative.

**20.** The method of claim 17, wherein said precursor contains a 7 position which can be hydroxylated.

**21.** The method of claim 17, wherein said precursor is chosen from the group consisting of 3-hydroxylated steroids, 3 $\beta$ -hydroxylated steroids, and steroids which has a 3-keto function.

**22.** The method of claim 17, wherein said precursor is a steroid with a structure chosen from the group consisting of structures of the androstane, androstene, pregnane, pregnene, cholane, cholene, cholesterol, ergostane, ergostene, testosterone and stigmastane type.

23. The method of claim 22, wherein said precursor is chosen from the group consisting of DHEA, testosterone, pregnenolone, pregnanolone, 25-hydroxycholesterol, 5- $\alpha$ -androstane-3 $\beta$ ,17 $\beta$ -diol and 5- $\alpha$ -androstene-3 $\beta$ ,17 $\beta$ -diol.

24. The method of claim 18, wherein the APAT activity of said yeasts has been rendered low or zero by inactivation of the *atf2* gene or by using an *atf2*<sup>-</sup> mutant.

25. The method of claim 17, wherein said yeasts also carry dehydrogenase activity.

26. The method of claim 25, wherein said dehydrogenase activity is a 17-dehydrogenase activity which produces a 17-hydroxylated derivative.

27. The method of claim 26, wherein said 17-dehydrogenase activity is carried by the *yi1124w* gene.

28. The method of claim 17, wherein the 17-dehydrogenase activity of said yeasts has been rendered low or zero.

29. The method of claim 17, wherein said yeasts are of the genus *Saccharomyces*.

30. The method of claim 17, wherein said *Cyp7b* gene is under the control of a yeast promoter chosen in the group consisting of CYC1, TEF1 and TDH3.

31. A yeast strain having zero 17-dehydrogenase activity by inactivation of the *yi1124w* gene.

32. A yeast strain transformed with a plasmid comprising an expression cassette expressing the *Cyp7b* gene.

33. A method preparing a medicinal product for the treatment of diseases of the central nervous system comprising the step of preparing a steroid by the method of claim 17.--

REMARKS

Entry of the foregoing amendments are respectfully requested.

Should the Examiner have any questions concerning the subject application, a telephone call to the undersigned would be appreciated.

Respectfully submitted,

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**Date: April 4, 2002**



METHOD FOR PREPARING MODIFIED STEROIDS  
BY YEAST FERMENTATION

5 The present invention relates to a novel method for producing hydroxylated and/or acetylated steroids by yeast fermentation.

10 Steroids, in particular cholesterol-derived steroids, are involved in many physiological processes, among which mention may be made of the regulation of carbohydrate and cholesterol levels in the circulating blood, the maintenance and development of muscle mass and the development of the central nervous system.

15 Among the drawbacks observed in the event of an imbalance in circulating steroid levels, mention may be made of the possible triggering of autoimmune diseases, such as lupus, of certain cancers, for example breast cancer, and of cardiovascular diseases, for example  
20 atherosclerosis. Problems with steroid regulation are also suspected in the case of the triggering of certain neurological diseases, such as Parkinson's disease or Alzheimer's disease.

25 The studies carried out, in particular by Professor Baulieu, on dehydroepiandrosterone, or DHEA, have shown the importance of steroids, in particular neurosteroids, in the development of the central nervous system, but also the possible impact of this type of  
30 steroid in all the closely related processes, including the phenomenon of aging (Baulieu and Robel, 1998, Proc. Natl. Acad. Sci., 95, 4089-4091).

35 It is therefore particularly advantageous to be able to have novel steroid derivatives, in particular of the neurosteroid family, which are involved in a very large number of physiological processes.

This is precisely one of the subjects of the present invention, namely the development of a method allowing access to novel steroid derivatives, in particular neurosteroids, using fermentation methods which permit  
5 high specificities and also considerable production yields.

The methods according to the present invention may, moreover, be used in order to obtain steroids which  
10 have a structure which is also known, but which, to date, were difficult to access using commercially acceptable methods.

The present invention is based on the demonstration of  
15 the fact that yeasts are capable of biologically converting, or bioconverting, precursor steroids, producing diverse hydroxylated and/or acetylated steroids which can, of course, if necessary, then be modified on such particularly reactive functions.

20 For this reason, the present invention relates in particular to a method for producing hydroxylated and/or acetylated steroids, comprising the steps according to which:

- 25
- yeasts are cultured on a medium comprising at least one precursor of such hydroxylated and/or acetylated steroids, and then
- 30
- the hydroxylated and/or acetylated steroids are isolated from the medium after bioconversion,

said method being characterized in that said yeasts are yeasts transformed so as to express the product of the  
35 *Cyp7b* gene.

Yeast naturally possesses an enzymatic acetylation activity encoded by the *atf2* gene and an enzymatic dehydrogenation activity encoded by the *yil124w* gene.

The enzyme encoded by the *atf2* gene (the sequence and activity of which are described in Cauet et al., 1999, Eur. J. Biochem., 261, 317-324 and in French patent application FR2774393 or at the address <http://genome-www.stanford.edu/Saccharomyces>) is acetyl coenzyme A-pregnenolone acetyltransferase, hereafter named "APAT". This enzyme allows the esterification, and more particularly the acetylation, of a steroidal precursor, such as for example a  $\Delta^5$  - or  $\Delta^4$  - $3\beta$ -hydroxysteroid, for instance pregnenolone, 17-hydroxypregnenolone, dehydroepiandrosterone (DHEA) or 4-pregnen- $3\beta$ -ol-20-one, for example. This esterification is preferably carried out on the OH function in position 3 of a said precursor. Expression of this activity in yeasts consequently makes it possible to produce derivatives of steroidal precursors which are completely or partially acetylated. Similarly, it is possible to chemically protect all or some of the OH functions of the precursor incorporated into the culture medium, such that the acetylation reaction may not take place on all or some of said functions. Such protection methods are well known and consist, for example, of addition of silane, of modification to an ester function, etc.

However, according to a particular embodiment of the present invention described hereafter, it is also possible to use yeast strains lacking the APAT enzymatic activity, such as in particular those described in Cauet et al. (1999, Eur. J. Biochem., 261, 317-324) or in French patent application FR2774393, the contents of which are incorporated into the present application by way of reference.

The *yil124w* gene was described during the yeast genome sequencing project, but its function had not then been determined. It is located on chromosome IX, at the coordinates 126204 to 127097. Its sequence is readily accessible to those skilled in the art in the database

for the *Saccharomyces* genome, located, for example, at the address <http://genome-www.stanford.edu/>. In the context of the studies of the present invention, it has been shown that the protein encoded by the *yil124w* gene

5 has dehydrogenase activity comparable to the activity of the 17 $\beta$ -hydroxysteroid dehydrogenase previously described in mammals (17BDH; Wu et al., 1993, J. Biol. Chem., 268, 12964-12969). This activity more specifically directs the reduction, to alcohol, of the ketone

10 function located, naturally or after chemical modification, in position 17 of certain steroidal precursors, such as DHEA for example. More particularly, in the case of the DHEA substrate, the enzymatic activity of the protein encoded by the *yil124w* gene leads to the

15 reduction of the ketone function of this steroid and to the formation of a 3,17-diol, hereafter referred to as "Diol". In addition, when made necessary, since the sequence of the *yil124w* gene is known, it is easy for those skilled in the art, familiar with techniques for

20 mutagenesis in yeast, to produce a yeast strain in which the *yil124w* gene is inactivated. For example, in accordance with the examples which follow, it is possible to obtain yeast strains for which the sequence encoding the *yil124w* gene has been knocked out (knock-

25 out technique) and the enzymatic activity suppressed.

The *Cyp7b* gene is known; it has already been described by Stapleton et al., (1995, J. Biol. Chem., 270, 29739-29745) or Rose et al. (1997, Proc. Natl. Acad.

30 Sci. USA, 94, 4925-4930). Its sequence is in particular described in patent application WO9612820 and is accessible from the Institute for Fermentation Osaka (IFO) under the accession code IFO 2031. The contents of those documents are incorporated into the present

35 application by way of reference. However, there was nothing to imply that this mammalian gene could be expressed, and more particularly expressed in its active form, by a yeast. In addition, there was nothing which made it possible to envision that such a yeast

would be capable of using a steroid as a substrate. Specifically, the *Cyp7b* gene is a gene isolated from mammals, in particular rats, mice and humans, in which the enzyme encoded by this gene belongs to the family  
5 of enzymes commonly named "cytochrome P450s", the active form of which contains heme (Poulos, 1988, Pharm. Res., 5, 67-75) and which are involved in the metabolic pathways for steroids (see Rose *et al.*, 1997, Proc. Natl. Acad. Sci. USA, 94, 4925-4930). The *Cyp7b*  
10 protein is a hydroxylase, and more particularly a 7-hydroxylase, which makes it possible to obtain 7-hydroxylated steroids, and more particularly 7 $\alpha$ -hydroxylated steroids. However, it is possible to envision the particular case for which the steroidal  
15 substrate is an equivalent of the known steroidal compounds, in which, by modification of the structure of one of the rings, the "position which is naturally 7" is transformed into a "position 6 equivalent". In this specific case, the *Cyp7b* protein would make it  
20 possible to obtain 6-hydroxylated steroids.

According to a preferred case of the invention, the action on the "Diol" compound described above of the *Cyp7b* enzyme expressed in the yeasts used allows the  
25 formation of a 3,7,17-triol, hereafter named "Triol". In addition, when the 3-OH function of said diol or of said triol is acetylated, reference will be made, respectively, to an "acetyl-diol" or "acetyl-triol".

30 The nature of the hydroxylated and/or acetylated steroids produced according to the method of the invention consequently depends on the possibility of the yeast expressing or not expressing the functions of acetylation encoded by the *atf2* gene and/or of dehydro-  
35 genation encoded by the *yil124w* gene, in combination with the possibility of said yeast expressing the *Cyp7b* hydroxylation enzyme.

The compounds the production of which according to the



non-fermentable carbon sources, such as for example glycerol or acetate.

According to a third variant, it is also possible to  
5 use yeast strains which have, naturally or after genetic manipulation, deacetylase activity. This activity has been described as acting on short-chain alcohol esters (rEF). The use of such a system would make it possible to deacetylate the products obtained  
10 by the APAT activity.

Finally, it is possible to use *atf2*<sup>-</sup> strains, i.e. strains in which the gene is not expressed or in which the product of this gene is inactive. More particu-  
15 larly, those skilled in the art are capable, via routine experiments, of preparing yeast strains lacking the APAT enzymatic activity, such as in particular those described in Cauet et al. (1999, Eur. J. Biochem., 261, 317-324) or in French patent application  
20 FR2774393, the contents of which are incorporated into the present application by way of reference.

Similarly, it may be advantageous to induce the activity of the *atf2* gene only at a certain moment of  
25 the method of the invention, for example sequentially to the expression of the *Cyp7b* gene. Thus, certain promoters have been described in yeast which are active only in the presence of an outside stimulus (Guarente et al., 1982, Proc. Natl. Acad. Sci., 79, 7410-7414) or  
30 when the yeasts are in the stationary phase (Panaretou et al., 1992, Eur. J. Biochem., 206, 635-640). The use of an *atf2* gene placed under the control of such a promoter consequently makes it possible to control the APAT activity in the yeast containing such a recombined  
35 gene.

Similarly, the presence in the yeast used in the method of the invention of an endogenous activity similar to the 17BDH activity of mammals (encoded by the *yil124w*





more particularly of the 3-hydroxylated steroids, and in particular the 3- $\beta$ -hydroxylated steroids, or the steroids which have a 3-keto function.

5 More particularly, without this list being limiting, in this invention, consideration will be given to a precursor selected from the group consisting of the steroids with a structure of the androstane, androstene, pregnane, pregnene, cholane, cholene,  
10 cholesterol, ergostane, ergostene, testosterone or stigmastane type, for example DHEA, testosterone, pregnenolone, pregnanolone, 25-hydroxycholesterol, 5- $\alpha$ -androstane-3 $\beta$ ,17 $\beta$ -diol or 5- $\alpha$ -androstene-3 $\beta$ ,17 $\beta$ -diol.

15 Of course, these compounds may also be obtained in acetylated form. In precursors which have a ketone function in position 17, this function can also undergo reduction, as has been described previously.

20 The invention may be implemented by using yeasts of various genera which may be selected, without this list being limiting, from *Candida*, *Yarrowia*, *Kluyveromyces*, *Schizosaccharomyces*, *Torulopsis*, *Pichia* and *Hansenula*.

25 A preferred embodiment of the invention will use yeasts of the genus *Saccharomyces*, for example *S. cerevisiae*.

30 The conditions under which these yeasts can be cultured are known to those skilled in the art; it is sufficient to add a substrate which is a precursor for the desired steroids to the culture medium in order to carry out the method according to the present invention.

35 The examples provided below show that some media are more favorable to the production of hydroxylated steroids than others; this is a parameter which can be readily optimized by those skilled in the art.



difficulty.

With regard to the *Cyp7b* gene, its nucleic acid sequence (Institute for Fermentation Osaka (IFO),  
5 accession code IFO 2031 or SEQ ID NO. 1) may be cloned under the control of a yeast promoter, such as CYC1 (Degryse et al. Yeast (1995), 11, pp. 629-640), TEF1 (Cotrelle et al., 1985, J. Biol. Chem., 260, 3090-3096) or TDH3 (Bitter and Egan, 1984, Gene, 32, 263-274), in  
10 order to allow its expression in the transformed yeasts.

The method of the invention also comprises a step according to which the steroids produced are isolated  
15 from the medium. Such a step does not constitute a crucial element of said method and may implement various techniques generally used in the domain of steroid purification (chromatography, HPLC, etc.).

20 The invention also relates to the novel hydroxylated steroids which may be, or which are, obtained using the method described above, and also to their use in the context of therapeutic or prophylactic applications, in particular as neurosteroids, for preparing a medicinal  
25 product intended to treat the human or animal body.

It also relates to compositions, in particular pharmaceutical compositions, containing such novel hydroxylated steroids. These pharmaceutical compositions also contain one or more support(s) acceptable  
30 from a pharmaceutical point of view. Such a support is preferably isotonic, hypotonic or weakly hypertonic and has a relatively low ionic strength, such as for example a sucrose solution. Moreover, such a support  
35 may contain any solvent or aqueous or partially aqueous liquid, such as nonpyrogenic sterile water. The pH of the formulation is also adjusted and buffered in order to correspond to the requirements of use *in vivo*. The formulation may also include a diluent, an adjuvant or

an excipient which is acceptable from a pharmaceutical point of view, and also solubilizing, stabilizing and/or preserving agents. For an injectable administration, preference is given to a formulation in aqueous, nonaqueous or isotonic solution. It may be provided in a single dose or in multiple doses in liquid form or dry form (powder, lyophilizate, etc.) which can be reconstituted extemporaneously with a suitable diluent.

10 The examples below will make it possible to understand the methods for constructing the vectors and for transforming the yeasts, but this involves, for the most part, technology which is already known to those skilled in the art. The vectors are described in 15 Degryse et al. (Yeast (1995), 11, pp. 629-640).

It is understood that the results below are given only by way of examples. Thus, the levels obtained, for example in the bioconversion manipulations, are only 20 given by way of indication and in no way represent a claim of the maximum capacity of the system under optimized conditions.

25 EXAMPLE 1: Construction of expression vectors for the *cyp7b* cDNA

The *cyp7b* cDNA is amplified using the oligo of sequence SEQ ID No. 1, thus introducing a SalI site (underlined) and a sequence of 4As before the ATG codon, and a 30 second primer of sequence SEQ ID No. 2 introducing a MluI site after the stop codon.

The first PCR product is subcloned, after SalI/MluI restriction, into the SalI/MluI site of the vector equivalent to pTG10164 (Degryse et al., Yeast (1995), 35 11, pp. 629-640). This produces a vector pTG14010 containing the *Cyp7b* gene under the control of the CYC1 promoter (Degryse et al. Yeast (1995), 11, pp. 629-640) in a 2 $\mu$  environment and with the G418R (neo) gene.

Next, the TEF1 promoter (Cotrelle et al., 1985, J. Biol. Chem., 260, 3090-3096) is cloned by ClaI/SalI exchange, which produces the vector pTG14011; the  
5 corresponding plasmids are described in the above-mentioned Yeast article.

The NotI expression block containing the CYC1 (respectively, TEF1) promoter was introduced into the  
10 2 micron-based plasmid pTG10042 (respectively, pTG10092) containing the URA3d gene, by the recombination method already described in the Yeast article, to give the plasmid pTG14012 (respectively, pTG14014). The pTG14015 plasmid was constructed in the same way as  
15 pTG14014, from pTG10220, which carries the URA3 gene, as a selectable gene.

The URA3d gene was chosen because it leads to selection for a very large number of copies in minimal medium.  
20 The URA3 gene produces an average number of copies and the selection pressure is reasonable in minimal medium.

The pTG14012, pTG14014 and pTG14015 plasmids were introduced by transformation into the FY1679-28c strain  
25 and the transformants were selected on a suitable minimal medium and then stored.

The table below provides a summary of the expression vectors for *cyp7b*:

30

<u>PLASMID</u>	<u>PROMOTER</u>	<u>SELECTABLE MARKER</u>
pTG14011	TEF1	G418R
pTG14012	CYC1	URA3d
pTG14014	TEF1	URA3d
pTG14015	TEF1	URA3

All the vectors contain the E. coli replicon and the origin of replication of the yeast 2 $\mu$  plasmid.

The yeasts used are:

5 FY1679-28c (Mata ura3-52 trp1 - 63 leu2 - 1 his3-  
200 GAL fen1), a Mata segregant from FY1679.

TGY202 and TGY206 are TRP1 derivatives obtained after transformation of the strains FY1679-28c and TGY186, respectively.

10

In TGY156, part of the *atf2* gene has been inactivated and replaced with URA3. TGY186 is a derivative of TGY156 in which the URA3 gene at the *atf2* locus has been replaced with TEF1::PGK1 in the form of an expres-  
15 sion block.

The strains are transformed using the lithium acetate procedure (Ito, et al., 1983, J. Bact. 153, 163-168) or electroporation (Nacken et al., 1994, Nucleic Acids  
20 Res, 22, 1509-10), and selected on a YNBG solid medium containing the suitable amino acids.

#### EXAMPLE 2: BIOCONVERSION

25 The cells are grown on a YNBG solid medium supplemented with the suitable amino acids. A preculture is effected for 24 hours at 28°C in a medium which has the same composition as the culture medium to be tested. The minimal medium (YNB and the carbon source) is supple-  
30 mented with 0.5% of casamino acids (in addition to the nutrients required for the strain).

When the growth medium contains glycerol (2%), glucose is also present at 0.1% in order to initiate the  
35 culture.

An aliquot of the preculture is used to inoculate 10 ml of culture medium with an optical density at 600 nm of 0.1 and DHEA is solubilized in a 50/50 tergitol/ethanol

mixture at 10 mg/ml (or added at the time indicated). At various time periods, a 500  $\mu$ l aliquot of the culture medium is taken and the steroids are extracted in order to assay them.

5

4 ml of dichloromethane are added to the culture medium, and the mixture is vortexed for 10 minutes and centrifuged for 3 minutes at 3 000 rpm. The organic phase is dried under a stream of nitrogen at 50°C.

10 500  $\mu$ l of dichloromethane are added to the residue, and the sample is vortexed rapidly and then dried as above. The residue is taken up in a 90/10 isopropanol/water mixture and transferred into injection tubes, which are sealed. The sample is analyzed by HPLC against  
15 standards composed of DHEA, 7-hydroxy-DHEA and acetyl-DHEA. Some standards also contain 5-androstene-3 $\beta$ ,17 $\beta$ -diol.

The various culture media which were used are YPG, YNBD  
20 (glucose at 2%) and YNBG (glycerol at 2%, glucose at 0.1%).

The results obtained are given in the examples below;

25 EXAMPLE 3: Demonstration of an intrinsic 17-dehydrogenase activity in yeast

Incubation of TGY202 and acetyl-DHEA for 49 hours in YNBD made it possible to isolate acetyl-diol, indicating  
30 dehydrogenase activity in the yeast.

The characterization of the product obtained made it possible to determine that this activity is similar to that of mammalian 17 $\beta$ -hydroxysteroid dehydrogenase  
35 (17BDH).

EXAMPLE 4: Identification of the *yil124w* gene encoding the 17BDH activity of the yeast *Saccharomyces cerevisiae*

5 The *yil124w* gene was amplified by PCR using the primers of sequences SEQ ID No. 3 and SEQ ID No. 4, which introduce, respectively, SalI and MluI restriction sites. The amplification product, after restriction with the SalI and MluI enzymes, was subcloned into the  
10 yeast vector pTG10851 to give the vector pTG14491. The *yil124w* gene is thus under the control of the yeast promoter TEF1 and of the yeast terminator PGK1. The origin of replication is 2-micron and the selectable marker is the G418-resistance gene.

15 After transformation of TGY206 with pTG14491, bioconversion is carried out for 24 hours in the YNBD medium, using DHEA as the substrate. The steroids present in the culture medium are analyzed:

20

	DHEA	Diol
TGY206	62	13
TGY206-pTG14491	27	52

The values are expressed in  $\mu\text{g/ml}$  of the steroid extracted from the culture medium.

25 These results confirm the 17BDH activity of the *yil124w* gene, and show that overexpression thereof makes it possible to produce reduced steroidal derivatives.

EXAMPLE 5: Generation of a knock-out mutant for the  
30 *yil124w* gene

The pTG14491 plasmid was restricted with PstI and NcoI (unique restriction sites in the coding sequence of *yil124w*) and the sticky ends were made blunt with T4  
35 DNA polymerase. The URA3 gene is then cloned into this plasmid such that the coding sequence of *yil124w* is



knocked out by this selectable marker URA3. The resulting plasmid, pTG14584, contains the URA3 gene in the same direction of translation as the TIL124w gene. After restriction of pTG14584 with SalI and MluI, the  
5 fragment released containing *yil124w* knocked out by URA3 is introduced into the TGY202 and TGY206 strains, and the recombinant colonies are selected on YNBD + LH, the selection being for prototrophy for uracil. The TGY279 and TGY282 strains, respectively, are thus  
10 obtained.

The TGY206 and TGY282 strains are incubated in the presence of DHEA or acetyl-DHEA in the YNBD medium for 24 h and the products obtained are measured.

15

Products	DHEA	Acetyl-DHEA	Diol	Acetyl-diols
DHEA substrate				
TGY206	56	0	43	0
TGY282	86	0	0	0
Acetyl-DHEA substrate				
TGY202	4	34	7	20
TGY282	16	46	0	0

Results expressed in  $\mu\text{g/ml}$  extracted from the culture medium.

20 It clearly appears, therefore, that the TGY282 strain is no longer capable of reducing the DHEA or acetyl-DHEA to, respectively, diol or acetyl-diols. This confirms the inactivation of the *yil124w* gene and also of its 17-dehydrogenase function.

25

Moreover, it may be observed that the yeast has an intrinsic activity of deacetylation of acetyl-DHEA to DHEA. Thus, identification of the gene responsible and its overexpression relative to the APAT activity will  
30 make it possible to produce steroids without using mutants of the *atf2* gene.

EXAMPLE 6: Use of the method according to the present invention to obtain 7-hydroxy-DHEA from DHEA using the TGY202 strain transformed with the expression plasmids for *cyp7b*

5 The TGY202 strain transformed with the pTG14012, pTG14014 or pTG14015 plasmid is incubated in the presence of DHEA (40 µg/ml) in a YPG medium, for 48 hours, and the presence of 7-hydroxy-DHEA  
10 (7αHO-DHEA) in the medium is evaluated.

The results are given in the table below:

<u>TGY202</u>	<u>7-HYDROXY-DHEA</u>
pTG14012	4.2
pTG14014	6.4
pTG14015	3.1

15 The presence of 7αHO-DHEA in the medium indicates that the *cyp7b* gene is expressed in an active form which allows the bioconversion of the DHEA to 7αHO-DHEA by the yeast. Thus, any potential substrate for Cyp7b may be hydroxylated *in vivo* by a yeast strain expressing  
20 *cyp7b*.

Moreover, these results indicate that the TEF1 promoter is more effective than CYC1, and that the selectable marker URA3d gives better results than the marker URA3.

25

EXAMPLE 7: Increase in the production of 7αHO-DHEA by bioconversion, by modulation of the fermentation conditions

30 TGY202-pTG14012 and TGY202-pTG14014 are incubated in the YNBD and YNBDG media for 48 hours, in the presence of DHEA (40 µg/ml) added to the medium immediately after inoculation or after 8 hours of incubation.

The results are given in the following table (as % of product accumulated).

	DHEA (40 µg/ml) added at t 0			
Medium	YNBD		YNBG	
	TGY202- pTG14012	TGY202- pTG14014	TGY202- pTG14012	TGY202- pTG14014
Acetyl-DHEA	20.1	9.6	65.2	55.8
Acetyl-diol				
Acetyl-triol				
DHEA	0	0	5.7	1.1
Diol	0	2.9	0	0
7αHO-DHEA	52.3	35.6	25.1	37.2
Triol	27.5	51.9	4.0	5.4
	DHEA (40 µg/ml) added at t 0 + 8 hours			
Acetyl-DHEA	3.0	2.0	25.1	0
Acetyl-diol				
Acetyl-triol				
DHEA	0	0	17.3	3.4
Diol	1.5	0	7.4	0
7αHO-DHEA	40.6	53.1	36.9	81.5
Triol	54.9	44.9	13.3	15.1

- 5 It is thus noted that the fermentation medium and the time at which the DHEA substrate is added have an influence on the selective production of 7αHO-DHEA, a delayed inoculation generally giving better results, and the YNBG medium being superior to the other media.

10

EXAMPLE 8: Preferential production of 7αHO-DHEA in the absence of acetylated derivatives by using KO strains for APAT activity

- 15 TGY206p-pTG14014 is incubated in YNBD in the presence of DHEA (100 µg/ml) for 49 hours.

The analysis of the products obtained indicates that

100% of 7 $\alpha$ HO-DHEA is recovered (91.7  $\mu$ g/ml).

When the culture medium is YNBG, a mixture of 7 $\alpha$ HO-DHEA (89%, 73.8  $\mu$ g/ml), triol (6%, 5  $\mu$ g/ml) and DHEA (5.3%, 4.4  $\mu$ g/ml) is obtained.

These observations indicate that, in the absence of APAT activity, the production of acetylated derivatives is avoided. On the other hand, the presence of the intrinsic 17BDH activity leads to a contaminating presence of Diol or Triol.

#### EXAMPLE 9: Preferential production of Triol

Incubation of TGY206p-pTG14014 in the presence of Diol (100  $\mu$ g/ml) for 48 hours leads to the production of 26.4  $\mu$ g/ml Triol (36%) of the final products, 74% of Diol remaining) in YNBG. The use of YNBD gives a bioconversion rate of 100%.

These results show that, by changing the fermentation medium, it is possible to channel the production of metabolites from a substrate. In particular, it may be readily envisioned that overexpression of the *yil124w* gene allows rapid and/or complete accumulation of reduced derivatives. By coupling this overexpression with that of *cyp7b*, it is thus possible to achieve virtually complete conversion to Triol, from DHEA as the starting substrate.

#### EXAMPLE 10: Preferential production of acetyl-DHEA using a yeast strain which is a KO strain for 17BDH activity

The TGY202 and TGY279 strains were incubated with DHEA for 24 hours in the YPD culture medium, and the steroids produced were measured.

	DHEA	Ac-DHEA	Diol	Ac-Diol
TGY202	1	84	14	1
TGY279	3	96	0	1

Results expressed as percentage of the total of the steroids extracted from the culture medium.

5

It clearly appears that the TGY279 strain virtually completely transforms the DHEA to acetyl-DHEA, in the absence of the 17BDH activity.

10 EXAMPLE 11: Exclusive production of 7 $\alpha$ HO-DHEA using the TGY282 strain, which is a KO strain for the APAT and 17BDH activities and expresses *cyp7b*

15 The TGY279 and TGY282 strains are transformed with the pTG14011 plasmid and selected on YPG + G418. Incubation of the transformed strains in the YPD medium for 24 hours, using DHEA as the substrate, makes it possible to measure the levels of steroids produced by these strains.

20

	DHEA	Ac-DHEA	Diol	Ac-Diol	7 $\alpha$ HO-DHEA
TGY279-pTG14011	3	49	1	0	47
TGY282-pTG14011	31	0	1	0	68

Results expressed as percentage of the total of the steroids extracted from the culture medium.

25 Although the bioconversion did not go to completion in this experiment, it is seen that the TGY282-pTG14011 strain produces exclusively 7 $\alpha$ HO-DHEA from DHEA, whereas a strain also containing the APAT activity produces not only 7 $\alpha$ HO-DHEA, but also acetyl-DHEA.

10/089803

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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

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U.S. National Serial No. :

Filed :

PCT International Application No. : PCT/FR00/02753

VERIFICATION OF A TRANSLATION

I, the below named translator, hereby declare that:

My name and post office address are as stated below;

That I am knowledgeable in the French language in which the below identified international application was filed, and that, to the best of my knowledge and belief, the English translation of the international application No. PCT/FR00/02753 is a true and complete translation of the above identified international application as filed.

I hereby declare that all the statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the patent application issued thereon.

Date: March 22, 2002

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03-09-2001

- 22 -

CLAIMS

1. A method for producing hydroxylated and/or acetylated steroids, comprising the steps according to which:
- yeasts are cultured on a medium comprising at least one precursor of such hydroxylated and/or acetylated steroids, and then
  - the hydroxylated and/or acetylated steroids are isolated from the medium after bioconversion,
- said method being characterized in that said yeasts are yeasts transformed so as to express the product of the *Cyp7b* gene.
2. The method as claimed in claim 1, for producing a hydroxylated steroid, characterized in that said yeasts have low or zero APAT activity, and/or in that said yeasts are cultured under conditions which are oxidative.
3. The method as claimed in either of claims 1 and 2, characterized in that said precursor contains a 7 position which can be hydroxylated.
4. The method as claimed in one of claims 1 to 3, characterized in that said precursor is a 3-hydroxylated steroid, preferably a 3 $\beta$ -hydroxylated steroid, or a steroid which has a 3-keto function.
5. The method as claimed in claims 1 to 4, characterized in that said precursor is chosen from the steroids with a structure of the androstane, androstene, pregnane, pregnene, cholane, cholene,

A/434

11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30 31 32 33 34 35 36 37 38 39 40 41 42 43 44 45 46 47 48 49 50

- 23 -

cholesterol, ergostane, ergostene, testosterone or stigmastane type.

- 5 6. The method as claimed in claim 5, characterized in that the precursor is chosen from the group consisting of DHEA, testosterone, pregnenolone, pregnanolone, 25-hydroxycholesterol, 5- $\alpha$ -androstane-3 $\beta$ ,17 $\beta$ -diol and 5- $\alpha$ -androstene-3 $\beta$ ,17 $\beta$ -diol.
- 10 7. The method as claimed in one of claims 2 to 6, characterized in that the APAT activity of the yeast has been rendered low or zero by inactivation of the *atf2* gene or by using an *atf2*<sup>-</sup> mutant.
- 15 8. The method as claimed in one of claims 1 to 7, characterized in that the yeast also carries dehydrogenase activity.
- 20 9. The method as claimed in claim 8, characterized in that the dehydrogenase activity is a 17-dehydrogenase which produces a 17-hydroxylated derivative.
- 25 10. The method as claimed in claim 9, characterized in that the 17-dehydrogenase activity is carried by the *yil124w* gene.
- 30 11. The method as claimed in one of claims 1 to 10, characterized in that the 17-dehydrogenase activity of the yeast has been rendered low or zero, in particular by inactivation of the *yil124w* gene or use of a *yil124w*<sup>-</sup> mutant.
- 35 12. The method as claimed in one of claims 1 to 11, characterized in that the yeast is of the genus *Saccharomyces*.

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En ce qui concerne les codes à deux lettres et autres abréviations, se référer aux "Notes explicatives relatives aux codes et abréviations" figurant au début de chaque numéro ordinaire de la Gazette du PCT.

(54) Title: METHOD FOR PREPARING STEROIDS MODIFIED BY YEAST FERMENTATION

(54) Titre: PROCEDE DE PREPARATION DE STEROIDES MODIFIES PAR FERMENTATION DE LEVURES

(57) Abstract: The invention concerns a novel method for producing hydroxylated and/or acetylated steroid comprising steps which consist in: culturing yeasts on a medium comprising at least a precursor of such hydroxylated and/or acetylated steroids; then isolating the hydroxylated and/or acetylated steroids from the medium after bioconversion. Said method is characterised in that said yeasts are yeasts transformed so as to express the product of *Cyp7b* gene. The invention also concerns the modified yeast strains.

(57) Abrégé: La présente invention concerne un nouveau procédé de production de stéroïde hydroxylé et/ou acétylé comprenant les étapes selon lesquelles: on cultive des levures sur un milieu comprenant au moins un précurseur de tels stéroïdes hydroxylés et/ou acétylés, puis on isole les stéroïdes hydroxylés et/ou acétylés du milieu après bioconversion, ledit procédé étant caractérisé en ce que lesdites levures sont des levures transformées de façon à exprimer le produit du gène *Cyp7b*. L'invention concerne aussi les souches de levures modifiées.

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Transgene MB

COMBINED DECLARATION AND POWER OF ATTORNEY

Attorney's Docket No

COUNTRY/INTERNATIONAL	APPLICATION NUMBER	DATE OF FILING (day, month, year)	PRIORITY CLAIMED
FRANCE	99/12410	October 5, 1999	YES <input checked="" type="checkbox"/> NO <input type="checkbox"/>
			YES <input type="checkbox"/> NO <input type="checkbox"/>

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1-0

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RESIDENCE	CITIZENSHIP	
POST OFFICE ADDRESS		

10/089803

## SEQUENCE LISTING

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<110> CAUET Gilles  
DEGRYSE Eric  
VICO Pedro  
LATHE Richard

<120> METHOD FOR PREPARATING MODIFIED STEROIDS BY YEAST FERMENTATION

<130> D18217

<150> FR99/12410

<151> 1999-10-05

<150> PCT/FR00/02753

<151> 2000-10-04

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<170> PatentIn Ver. 2.1

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to isolate gene yil124w of saccharomyces  
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<110> TRANSGENE SA

<120> METHOD FOR PREPARING MODIFIED  
STEROIDS BY YEAST FERMENTATION

<130> D18217

<150> FR 99 12410

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- 2 -

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